MOLECULAR AND CELLULAR BIOLOGY, Feb. 1989, p. 787–797
0270-7306/89/020787-11$02.00/0

Vol. 9, No. 2

Structure, Chromosome Location, and Expression of the Mouse Zinc Finger Gene *Krox-20*: Multiple Gene Products and Coregulation with the Proto-Oncogene c-*fos*

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Received 29 August 1988/Accepted 8 November 1988

We have analyzed the structure and the regulation of *Krox-20*, a mouse zinc finger-encoding gene which is transiently activated following serum stimulation of quiescent fibroblast cells in culture. The gene is localized on chromosome 10, band B5, in the mouse, and the homologous human gene also maps to chromosome 10 (region q21.1 to q22.1). Alternative splicing of the 5′-most intron of the *Krox-20* gene gives rise to mRNAs encoding putative zinc finger proteins with different N termini. The first exon contains a sequence element with strong similarity to the c-*fos* proto-oncogene serum response element (SRE). This element can functionally substitute for the c-*fos* SRE, and it binds the same nuclear protein. It is probably responsible for the serum induction of *Krox-20*, possibly in combination with a weaker SRE located in the 5′-flanking region of the gene. Our findings suggest that c-*fos*, *Krox-20*, and a number of immediate-early serum response genes are coregulated and that the SRE and its cognate protein are essential components of this regulatory pathway.

Mitogenic stimulation of eucaryotic cells leads to DNA replication and cellular proliferation, presumably via a cascade of both transcriptional and posttranscriptional regulatory events. The identification of these events and their molecular characterization are essential for the understanding of both the mechanisms controlling normal cell growth and oncogenic transformation. Mouse NIH 3T3 fibroblasts constitute a convenient system for studying mitogenic stimulation since they can be brought to a quiescent state (G0) by serum deprivation and, upon subsequent exposure to serum or purified growth factors, they reenter the G1 phase and resume proliferation (54). Serum stimulation of quiescent fibroblasts leads to rapid activation of a number of so-called immediate-early response genes, including the proto-oncogenes c-*fos* and c-*myc*, whose expression has been demonstrated to be essential to reach DNA synthesis (33, 35, 52, 60). More than 80 unrelated cDNAs corresponding to immediate-early genes have been isolated in different laboratories (1, 14, 38, 39, 43). The recent observation that some of these genes encode potential transcription factors (10, 15, 37, 42, 56, 61, 62, 66; M. Zerial et al., unpublished data) is of particular interest since they might therefore represent members of the hierarchy of regulatory genes controlling cell proliferation. Equally interesting are the analysis of the mode of activation of these immediate-early genes and the possibility that all such genes are regulated through a common molecular mechanism.

We have recently isolated cDNAs corresponding to two immediate-early genes, *Krox-20* and *Krox-24* (10, 42). These genes are activated very rapidly and transiently following serum stimulation of NIH 3T3 cells, with kinetics similar to those of c-*fos* activation. De novo protein synthesis is not required for induction as judged by treatment of the cells with the protein synthesis inhibitor cycloheximide (10, 42). Indeed, cycloheximide leads to superinduction of the genes, due to both stabilization of the mRNA and prolongation of the period of transcription (1, 10, 42). Both *Krox-20* and *Krox-24* encode proteins containing three zinc finger domains (10, 42, 48, 66). Zinc fingers are potential DNA-binding motifs which are present in many eucaryotic transcription regulatory proteins and were first recognized in *Xenopus laevis* transcription factor IIIA (for a recent review, see reference 22). They consist of units of 28 to 30 amino acids and contain two cysteines and two histidines at invariant positions (7, 49). The mouse genome contains a large family of genes encoding zinc fingers that includes *Krox-20* and *Krox-24* (9, 12). The zinc fingers of the putative *Krox-20* and *Krox-24* proteins are almost identical and are similar to the three fingers of the DNA-binding transcription factor Sp1 (10, 36, 42). These observations strongly suggest that Krox-20 and Krox-24 are DNA-binding factors, that they recognize the same DNA sequence, and that they might regulate the transcription of other genes.

Here we present the characterization of the mouse genomic region encoding *Krox-20* mRNA. The structure and chromosomal location of the gene have been established. *Krox-20* mRNA is differentially spliced, and the gene might encode at least two finger proteins. In addition, we show that the parallel activation of *Krox-20* and c-*fos* following serum stimulation of NIH 3T3 cells is due to coregulation involving the same *cis*- and *trans*-acting elements. This molecular mechanism is likely to be of general significance and to constitute the basis for the activation of a number of immediate-early response genes.

MATERIALS AND METHODS

Plasmid construction, oligonucleotides, and DNA binding gel electrophoresis assay. All plasmid DNAs were constructed and prepared by the standard techniques of Maniatis et al. (44). pF261 carries a 5′ truncation of the human c-*fos* gene to position −261 bounded by a dodecamer *Eco*RI linker (67). Serum response element (SRE)-containing plas-

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mids were constructed by inserting synthetic oligonucleotides into the pF261 *Eco*RI site. After cloning, the nucleotide sequence of the oligonucleotide was verified and its orientation was determined by using the supercoiled-plasmid-sequencing procedure (11). The sequences of the synthetic oligonucleotides were as follows: pF261/27+ (SRE-1), (5'-AATTCCTCAGTCCATATATGGGCAGCG-3') annealed to 5'-AATTCGCTGCCCATATATGGACTGAGG-3'); pF261/37+, (5'-AATTCCTCAGTCCATATATGGGCAGCGACGT CACGGG-3') annealed to (5'-AATTCCCGTGACGTCGCT GCCCATATATGGACTGAGG-3'); and pF261/29+ (SRE-2), (5'-AATTCTCTTCTCCTTTTTTGGAAAGTCG-3') annealed to (5'-AATTCGACTTTCCAAAAAAGGAGAAG AG-3'); pF261/124+ was constructed by inserting a 124-base-pair (bp) *Alu*I fragment containing *Krox-20* SRE-1 after conversion into an *Eco*RI fragment by linker addition. pF261D+ contains a single c-*fos* SRE inserted into the *Eco*RI site (67, 68). DNA binding gel electrophoresis assays were performed as described previously (68). The probe was the *Eco*RI insert of pF261/124+ derived from the 124-bp *Krox-20 Alu*I DNA fragment. HeLa cell nuclear extracts prepared by the method of Dignam et al. (19) were kindly provided by A. Lamond. The *Krox-20* SRE-1 and SRE-2 double-stranded oligonucleotides used in the competition binding experiment were those described above. The other oligonucleotides were as follows: c-*fos* SRE, (5'-AATTGGA TGTCCATATTAGGACATCT-3' annealed to 5'-AATTAG ATGTCCTAATATGGACATCC-3'; and AP-2 binding site, 5'-GAACTGACCGCCCGCGGCCCGTGT-3' annealed to 5'-ACACGGGCCGCGGGCGGTCAGTTC-3').

Cell culture, cell transfection, and RNA and DNA extraction. NIH 3T3 cells were maintained in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. Serum deprivation and subsequent serum stimulation were performed as described previously (10). Cycloheximide was used at a concentration of 10 μg/ml. Cytoplasmic RNA was isolated as described previously (23, 44). Poly(A)$^+$ RNA was selected by oligo(dT)-cellulose (Collaborative Research, Inc., Waltham, Mass.) column chromatography (44). Mouse liver genomic DNA was extracted as described previously (44).

NIH 3T3 cell transfection and serum stimulation assays of transfected genes were performed as described previously (67, 68), except for RNA preparation. Total cellular RNA was prepared as follows. Cells were rinsed twice with cold phosphate-buffered saline and lysed by the addition of a solution containing 50 mM Tris hydrochloride (pH 7.4), 150 mM NaCl, 20 mM EDTA, 1% (vol/vol) *N*-lauryl-sarcosine, and 200 μg of proteinase K per ml. The lysate was vortexed thoroughly to reduce viscosity and then mixed with CsCl (1 g/ml of lysate) and underlaid with 2.5 ml of 5.7 M CsCl (30). After 18 h of centrifugation at 27,000 rpm in a Beckman SW40 rotor at 20°C, the RNA was recovered as a DNA-free pellet, dissolved in 200 μl of 300 mM sodium acetate (pH 5.4), and precipitated with 3 volumes of ethanol.

Cosmid library screening and cosmid mapping. Two mouse genomic cosmid libraries (gifts of A.-M. Frischauf) were screened for clones containing the *Krox-20* gene. The libraries had been generated by cloning genomic fragments resulting from partial digestion with *Mbo*I into the vectors pcos2EMBL and pcos4EMBL (21). For each library, five filters (GeneScreen; 20 cm by 20 cm) with approximately 10^5 colonies per filter were screened in duplicate with a *Krox-20* cDNA probe as follows. The probe was labeled to a specific activity of approximately 10^9 cpm/μg by the method of Feinberg and Vogelstein (24). Hybridizations were performed as described previously (13), with a probe concentration of 0.5×10^6 to 1×10^6 cpm/ml. Six cosmid clones were isolated, and three clones covering the *Krox-20* locus over more than 80 kilobases (kb) were analyzed in more detail. Restriction mapping of the genomic regions contained in the cosmids was performed as described previously (57, 70, 71).

cDNA library screening and Northern and Southern blotting. The lambda NM1149 cDNA library (1) was screened as described previously (44) by using 10^6 cpm of probe per ml (specific activity, approximately 10^9 cpm/μg) labeled with ^{32}P as described previously (24). Northern (RNA) blotting and Southern blotting were performed as described previously (10).

DNA sequencing and sequence analysis. A 4-kb *Bam*HI DNA fragment isolated from cosmid 8/4 and containing the 5' part of *Krox-20* and 5'-flanking sequences was subcloned in the *Bam*HI site of pUC19. Various restriction fragments derived from this plasmid were subsequently cloned into M13-derived vectors. Single-stranded DNA was prepared (47), and the nucleotide sequence was established by using the Sequenase procedure (United States Biochemical Corp., Cleveland, Ohio). In addition, a *Bgl*II-*Apa*I fragment covering the 3' part of the coding sequence was also sequenced by using as primers synthetic oligonucleotides whose sequences were designed from the cDNA sequence. The entire sequence was read on both strands. Nucleotide and amino acid sequences were analyzed by using the University of Wisconsin Genetics Computer Group Sequence Analysis Software Package, version 5 (17).

Primer extension analysis and S1 and RNase mapping. Primer extension analysis was performed by the method of Hernandez and Keller (34). A 5-μg amount of poly(A)$^+$ RNA was suspended in 20 μl of 10 mM Tris hydrochloride (pH 7.4)–300 mM NaCl–0.2 mM EDTA–50% (vol/vol) formamide and mixed with an excess of single-stranded 5'-end-labeled DNA primer. After being heated at 80°C for 5 min, probe and RNAs were allowed to hybridize for 16 h at 30°C. After primer extension, the complementary radioactive cDNA products were analyzed on a 0.5-mm-thick, 8% polyacrylamide sequencing gel. The single-stranded primer was prepared from a *Dde*I-*Aat*II restriction fragment localized between positions 305 and 253 in the *Krox-20* nucleotide sequence (see Fig. 3). The procedure for S1 mapping was as described above, except that the DNA-RNA hybrids were digested with nuclease S1 (400 U/ml) under the conditions recommended by the manufacturer (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Transcript mapping by RNase protection was performed by the methods of Zinn et al. (72) and Treisman (67). Probes were synthesized by the methods of Zinn et al. (72) and Melton et al. (46). The two RNA probes used for the mapping of *Krox-20* RNA 5' ends were obtained by transcription of restriction fragments cloned in the vector pGEM-1 (Promega Biotec, Madison, Wis.); probe Pv1.5, a *Pvu*II-*Sac*I restriction fragment localized between positions −561 and 152; and probe Pv1.3, an *Eco*RI-*Sac*I restriction fragment localized between positions −40 and 152. The RNA preparations used for S1 mapping and RNase protection experiments were different. The RNA probes used in the transient stimulation assay and the corresponding nuclease-resistant products were as follows (67). The human c-*fos* probe is derived from an *Xmn*I-*Bss*HII fragment (positions +488 to −100), and the major protected fragment, corresponding to exon 1, is 296 nucleotides long. The human alpha-globin RNA probe is derived from a

222-nucleotide-long *Ava*I-*Bam*HI restriction fragment, yielding an exon 1-protected fragment of 132 nucleotides.

Gene mapping by in situ hybridization. In situ hybridization experiments were performed by using metaphase spreads of lymphocytes. In the case of the localization to mouse chromosomes, the lymphocytes were derived from a WMP male mouse in which all of the autosomes except autosome 19 were in the form of metacentric robertsonian translocations. Concanavalin A-stimulated lymphocytes were cultured at 37°C for 72 h; 5-bromodeoxyuridine was added for the final 6 h of culture (60 μg/ml) to ensure a chromosomal R banding of good quality. Human lymphocytes were stimulated with phytohemagglutinin. A pGEM1 clone containing the 2.4-kb AC16 cDNA of *Krox-20* was labeled with tritium by nick translation to a specific activity of 1.1×10^8 dpm/μg. The radiolabeled probe was hybridized to metaphase spreads of chromosomes at a final concentration of 12 ng/ml as described previously (45). After being coated with nuclear track emulsion (Kodak NTB_2), the slides were exposed for 13 days at 4°C. To avoid any slipping of silver grains during the banding procedure, chromosome spreads were first stained with buffered Giemsa solution and metaphase spreads of chromosomes were photographed. Chromosomes were then R banded by the fluorochrome-photolysis-Giemsa solution method and metaphase spreads of chromosomes were rephotographed before analysis.

RESULTS

Chromosomal location. The chromosomal location of the *Krox-20* gene was first determined in the mouse genome. A total of 100 metaphase cells were examined after in situ hybridization with a 2.4-kb *Krox-20* cDNA probe, AC16, corresponding to the 3′ three-fourths of the mRNA (10). There were 144 silver grains associated with chromosomes, and 34 (23.6%) were located on chromosome 10 (Fig. 1A). The distribution of grains on this chromosome was not random; 82% of them mapped to the B4 to C3 region of chromosome 10, with a maximum in the B5 band (Fig. 1B). Therefore, the most probable location of the gene was the distal part of the B band of chromosome 10. The assignment of *Krox-20* to chromosome 10 was independently confirmed by genetic localization of the gene (M. Bucan, personal communication).

The similarity between the mouse *Krox-20* gene and its human homolog allowed detection of the latter gene with the AC16 cDNA probe in Southern blotting experiments (data not shown). Therefore, we also used this probe to determine the chromosomal location of the human homolog of *Krox-20*. A total of 100 metaphase cells were examined. Of the grains associated with chromosomes, 20% were located on chromosome 10, and 70% of those grains mapped to the region q21.1 to q22.1 (data not shown). In this case, the most probable location of the gene was 10q21.3. This is consistent with the position of the mouse gene, since two other genes have been localized on chromosome 10 in both humans and the mouse: the genes encoding pyrophosphatase (precise location of the human gene, 10q11.1 to q24) and hexokinase (precise location of the mouse gene, 10B4) (64).

Isolation and restriction mapping of *Krox-20* genomic sequences. Two mouse genomic cosmid libraries (generous gifts of A.-M. Frischauf) were screened with the AC16 cDNA probe. Six independent positively hybridizing clones were isolated, and three of them, designated 8/4, 13/13, and 16/13, were analyzed in further detail. Preliminary restriction mapping of the cosmid DNAs indicated that they overlapped

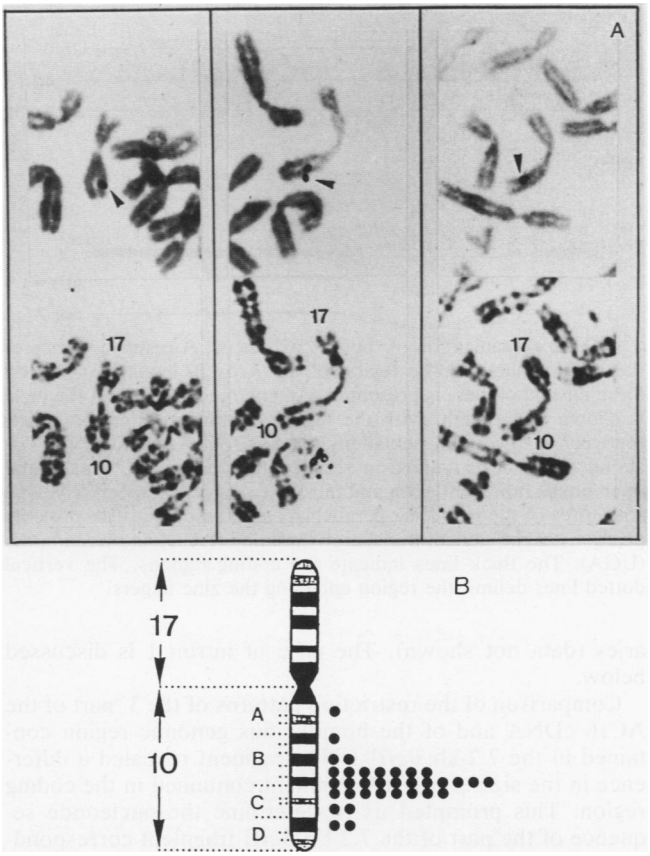

FIG. 1. Chromosomal localization of the *Krox-20* gene in the mouse genome. (A) Three examples of partial WMP mouse metaphase, showing the specific site of hybridization to chromosome 10. In the upper half of each panel the positions of silver grains are indicated by arrows on Giemsa-stained chromosomes. The bottom half of each panel shows subsequent identification of the chromosomes by R-banding. (B) Diagram of WMP mouse robertsonian (10 and 17) chromosome, indicating the distribution of grains on 34 labeled chromosomes 10.

(Fig. 2 and data not shown), and Southern blotting analysis showed that the cDNA spanned two *Bam*HI fragments of 4 and 10.4 kb (Fig. 2 and data not shown). Fragments of similar size were detected in Southern blotting analysis of *Bam*HI-digested mouse genomic DNA (data not shown), suggesting that *Krox-20* DNA was not rearranged during the cloning procedure. Detailed restriction mapping of the cosmid DNAs was performed (Fig. 2). The 4-kb *Bam*HI fragment containing the 5′ part of the gene and the slightly overlapping 7.2-kb *Bgl*II fragment containing the 3′ part of the gene were subcloned from cosmids 8/4 and 16/13, respectively, for further analysis (Fig. 2).

Structure of the *Krox-20* gene. Comparison of the restriction map of the 4-kb *Bam*HI DNA fragment with that of pEX2.8, a 2.8-kb cDNA clone of *Krox-20* (10), suggested that the 5′ part of the *Krox-20* gene contained two introns (data not shown). The nucleotide sequence of the 4-kb *Bam*HI fragment was determined, except for an approximately 1-kb region internal to the second intron (Fig. 3). Comparison of this sequence to that of the pEX2.8 cDNA clone allowed the precise localization of the two introns. Within the common regions, the genomic and cDNA nucleotide sequences were identical. The positions of the splice junctions of intron 2 were verified by RNase mapping by using uniformly labeled probes covering each of the bound-

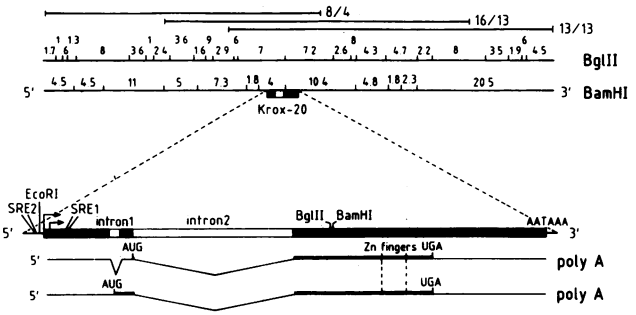

FIG. 2. Organization of the *Krox-20* locus. A restriction map of the approximately 80-kb region of the *Krox-20* locus, covered by three cosmid clones, is presented. An enlarged diagram of the gene is shown underneath, with the region of transcriptional initiation (between arrows) and polyadenylation (AATAAA) and of the *cis*-acting sequences involved in serum induction (SRE). Closed and open boxes represent exon and intron sequences, respectively. The structures of the two *Krox-20* mRNAs are presented, with possible positions of the sites of translation initiation (AUG) and termination (UGA). The thick lines indicate the coding regions. The vertical dotted lines delimit the region encoding the zinc fingers.

aries (data not shown). The case of intron 1 is discussed below.

Comparison of the restriction patterns of the 3′ part of the AC16 cDNA and of the homologous genomic region contained in the 7.2-kb *Bgl*II DNA fragment revealed a difference in the size of a DNA fragment contained in the coding region. This prompted us to determine the nucleotide sequence of the part of the 7.2-kb *Bgl*II fragment corresponding to the *Krox-20* coding sequence. Indeed, a 29-bp deletion was observed in the cDNA and accounted for the difference in restriction patterns. In addition, three single-nucleotide differences were revealed which corresponded to sequencing errors in the cDNA. The deletion in the cDNA is not likely to correspond to an intron for the following reasons. (i) Its boundaries have no similarity to intron/exon boundaries. (ii) RNase mapping analysis of RNA from serum-stimulated cells showed that at least most of the *Krox-20* mRNA molecules do contain the genomic sequence deleted in AC16. (iii) Other *Krox-20* cDNAs analyzed, including pEX2.8, do not contain the deletion. The presence of the deletion in AC16 is likely to be due to the prolonged cycloheximide treatment of the cells which were used for preparing the cDNA. Indeed, we have noted recently that a number of cDNA clones derived from the same RNA preparation present abnormalities (data not shown). The corrected nucleotide sequence of the gene and the amino acid sequence of its putative product are presented in Fig. 3. No difference in restriction pattern was detected between the 3′ part of the pEX2.8 cDNA and the corresponding genomic region, suggesting that there was no additional intron in this region (data not shown).

The position of the 5′ end of the gene was determined by a combination of primer extension and RNase mapping analyses performed on cytoplasmic RNA extracted from NIH 3T3 cells treated with fetal calf serum and cycloheximide for 4 h. The results of the primer extension experiment suggested that the 5′ end of the *Krox-20* mRNA was heterogeneous and that the sites of initiation of transcription were spread over a region of approximately 80 bp (Fig. 4, lanes 1 to 4). This was confirmed by RNase mapping using two different, uniformly labeled RNA probes covering the presumed 5′ end of the gene (Fig. 4, lanes 5 to 11). The sizes of the protected RNA fragments revealed by RNase mapping and common to both probes were completely consistent with the results of the primer extension experiment, except that the most 5′ initiation site detected by primer extension was not observed in the RNase mapping analysis (Fig. 4). This allowed a precise localization of the sites of initiation of transcription (Fig. 3). The region immediately upstream of these sites does not contain any sequence with good similarity to the TATA box (Fig. 3). This might explain the heterogeneous 5′ end, since the TATA box is involved in precise positioning of the site of transcription initiation (4, 8, 18, 27, 31, 32, 53) and since genes without a TATA box usually have heterogeneous initiation sites (59). The information obtained on the structure of the *Krox-20* gene is summarized in Fig. 2 and 3.

***Krox-20* is subject to differential splicing.** The short (100-bp) 5′ intron of *Krox-20* (intron 1) is located immediately upstream from the first ATG initiation codon of the pEX2.8 cDNA, and its nonexcision would result in an extension of the open reading frame (Fig. 3). We therefore found it important to determine whether this intron might be subject to differential splicing. Northern blotting analysis using an intron-1-specific probe detected a 3.2-kb mRNA in serum-stimulated fibroblasts (data not shown). An RNA of approximately the same size had been revealed by the AC16 cDNA probe (10). Therefore, it appeared likely that intron 1 was subject to alternative splicing but that the Northern blotting procedure did not allow distinction between the two RNA molecules generated. The intron probe was then used to screen the original lambda NM1149 cDNA library from which the AC16 DNA had been isolated (1, 10). Nine clones among approximately 300,000 screened plaques hybridized to the probe on duplicate filters. Six clones were purified, and clone pG122, carrying the longest insert, 1.7 kb, was characterized in further detail. Restriction mapping and determination of the nucleotide sequences of both extremities of this cDNA indicated that it contained an unspliced intron 1. Otherwise its structure was identical to that of the pEX2.8 cDNA with the second intron spliced out, except that pG122 was shorter at both the 5′ and 3′ ends (Fig. 3). These results definitively established the existence of two *Krox-20* mRNAs, differing by the presence of intron 1. They are referred to here as spliced and unspliced mRNAs.

To determine the relative amounts of spliced and unspliced RNA, we performed an S1 mapping experiment with a single-stranded DNA probe covering the 3′ border of the first intron and labeled at its 5′ end (Fig. 5B). Cytoplasmic RNA from serum-stimulated cells but not from quiescent cells led to specific protection of the probe (Fig. 5A). Two clusters of protected fragments of 115 to 120 nucleotides and 75 to 78 nucleotides were observed. This was consistent with the length expected for the protected fragment for the unspliced RNA (115 nucleotides) and spliced RNA (75 nucleotides). The relative amount of protected fragment corresponding to spliced RNA versus that corresponding to unspliced RNA was very low, in the range of 1 to 2% as estimated by densitometer scanning of the autoradiogram.

In conclusion, our data indicated that both spliced and unspliced *Krox-20* mRNAs were present in serum-stimulated cells and that most of the mRNA was unspliced. Since the nonexcision of intron 1 extended the open reading frame, they suggested that *Krox-20* encoded at least two finger proteins. The protein encoded by the unspliced mRNA carried a 50-amino-acid extension at its N terminus (Fig. 2 and 3).

***Krox-20* and c-*fos* have related *cis*-acting regulatory sequences.** Serum stimulation of NIH 3T3 cells resulted in

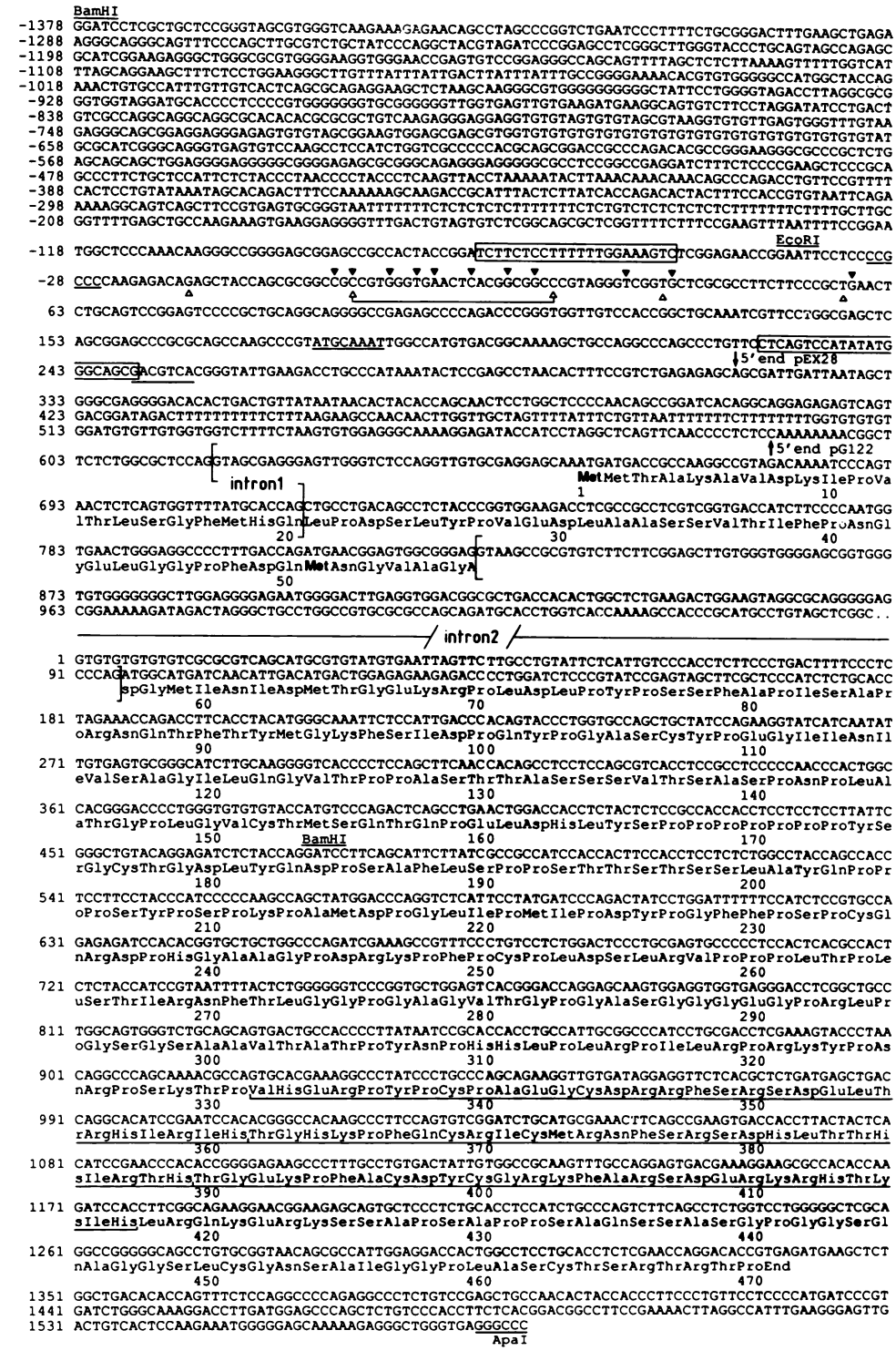

FIG. 3. Nucleotide sequence of the *Krox-20* gene and 5′-flanking region. Along the sequence are indicated the sites of transcriptional initiation as determined by RNase mapping (solid vertical arrowheads) and primer extension (open vertical arrowheads), the locations of SRE sequences (boxes), and the locations of potential binding sites for the transcription factors Sp1 and AP-1 and the octamer binding factor (underlined); the positions of intron/exon boundaries (brackets), and the positions of the 5′ ends of two cDNAs, pEX2.8 and pG122. The 5′ part of the nucleotide sequence has been numbered taking as origin the most 5′ site of initiation of transcription as detected by RNase mapping. The numbering of the 3′ part of the sequence of the fragment has been reinitiated arbitrarily since the complete sequence of the second intron has not been established. The amino acid sequences of the putative *Krox-20* proteins are shown under the nucleotide sequence. The two methionine residues shown in boldface type correspond to the first methionines encoded by the unspliced or spliced mRNAs. The amino acid sequences of the three zinc finger domains are underlined. The nucleotide sequence of the complete 3′-noncoding region of the gene has been reported previously (10). The 3′ end of the pG122 cDNA is within the 3′-noncoding region of the gene, at position 1909 of the cDNA (10).

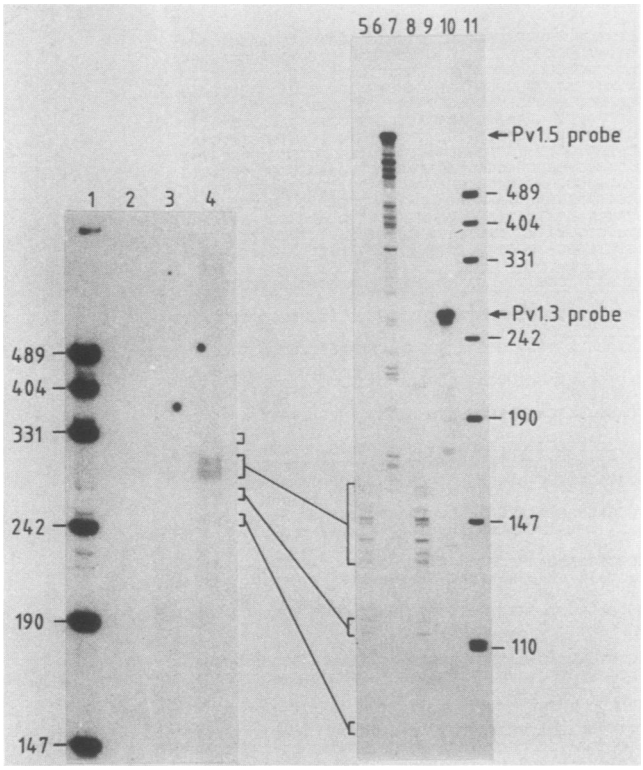

FIG. 4. Mapping of *Krox-20* mRNA 5′ end. Cytoplasmic RNA was extracted from quiescent NIH 3T3 cells or from cells serum stimulated in the presence of cycloheximide for 4 h. Primer extension or RNase mapping analyses were performed on 5 μg of poly(A)$^+$ RNA. The primer and the templates for the RNA probes are described in Materials and Methods. The products of the primer extension (lanes 1 to 4) and of the RNase mapping analyses (lanes 5 to 11) were analyzed by electrophoresis on an 8% polyacrylamide sequencing gel. Lanes: 1, DNA size marker; 2, extension in the presence of 5 μg of tRNA only; 3, extension with RNA from quiescent cells; 4, extension with RNA from serum-stimulated cells; 5 and 6, analysis of RNA from serum-stimulated cells and tRNA, respectively, with the Pv1.5 RNA probe; 7, intact probe Pv1.5; 8 and 9, analysis of RNA from serum-stimulated cells and tRNA, respectively, with the Pv1.3 RNA probe; 10, intact probe Pv1.3; 11, DNA size marker. The autoradiograms were exposed for 48 h. The correspondence between the extension products and the protected RNA fragments is indicated.

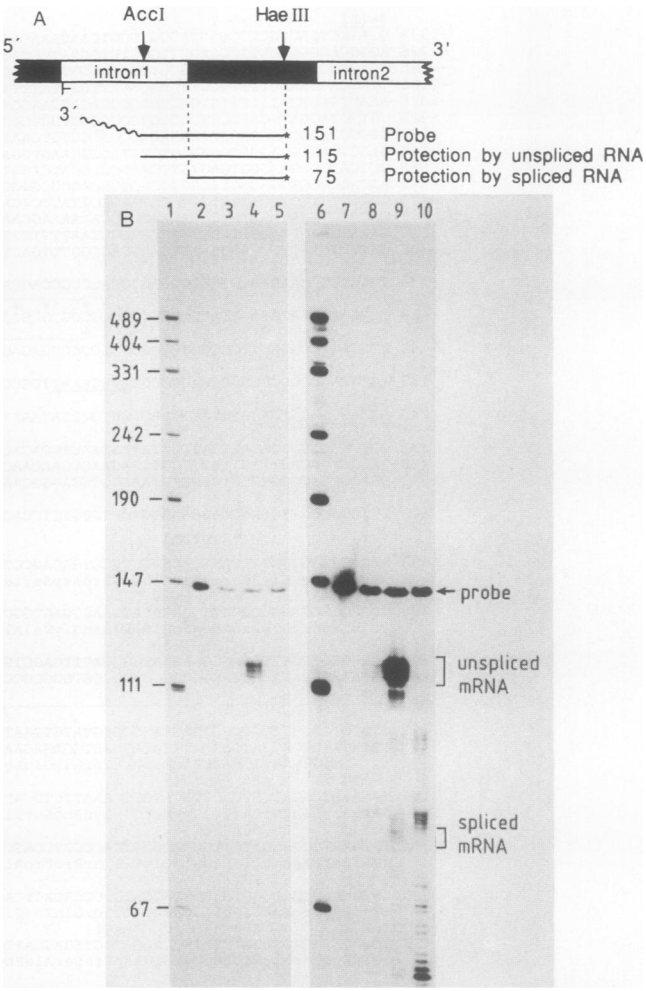

FIG. 5. Alternative splicing of *Krox-20* mRNA. (A) Description of the S1 mapping probe and of the protected fragments. The single-stranded 151-nucleotide DNA probe covering the intron 1/exon 2 boundary was labeled with ^{32}P at its 5′ end. It contained 36 nucleotides derived from the pUC19 polylinker at its 3′ end. (B) S1 mapping of the 3′ boundary of *Krox-20* intron 1. Cytoplasmic RNA was prepared from quiescent NIH 3T3 cells or from cells serum stimulated in the presence of cycloheximide for 4 h. S1 mapping analysis was performed on 5 μg of poly(A)$^+$ RNA, and the digestion products were fractionated by electrophoresis on an 8% polyacrylamide sequencing gel. Lanes: 1 and 6, DNA markers; 2 and 7, undigested probe; 3 and 8, analysis of RNA from quiescent cells; 4 and 9, analysis of RNA from serum-stimulated cells; 5 and 10, analysis of tRNA (5 μg). Lanes 1 to 5 and lanes 6 to 10 correspond to 5- and 76-h exposures, respectively, of the same gel. The positions of the protected fragments corresponding to unspliced and spliced mRNAs are indicated.

transcriptional activation of the *Krox-20* and c-*fos* genes, with similar kinetics (1, 10), suggesting that the two genes might be subject to coordinate regulation and that they might share particular transcriptional activation mechanisms. In the case of c-*fos*, a *cis*-acting DNA element called the SRE has been shown to be necessary and sufficient for transcriptional activation of the gene by serum or epidermal growth factor (25, 67, 68). This element consists of a 22-bp partially palindromic sequence which binds a cellular protein, the serum response factor (SRF) (29, 50, 68, 69). To investigate whether *Krox-20* and c-*fos* contain identical *cis*-acting elements, we compared the nucleotide sequences of the 5′-flanking region of the mouse c-*fos* gene and of the 5′-flanking and 5′-untranslated region of *Krox-20* by using the bestfit program of the University of Wisconsin Genetics Computer Group. This analysis revealed one region of significant similarity located between positions −312 and −285 in the mouse c-*fos* gene (positions −320 to −293 in the human c-*fos* gene) and between positions 228 and 255 in the *Krox-20* gene (Fig. 3). As shown in Fig. 6A, the shared region included the c-*fos* SRE sequence and another palindromic sequence located outside of the SRE. The second shared palindrome is similar to the core motif of the cyclic AMP-responsive element (CRE) shown to be involved in the cyclic AMP activation of preproenkephalin and somatostatin genes (16, 51). Finally, another 9-bp sequence, complementary to the 3′ half of the palindromic *Krox-20* SRE-like sequence, was located 14 bp downstream from the *Krox-20* CRE-like sequence (Fig. 6A).

To investigate whether the SRE-like sequence of *Krox-20* might be functional, we proceeded in the following way.

A

Krox-20 CTCAGTCCATATATGGGCAGCGACGTCACGGGTATTGAAGACCTGCCCATAA

c-*fos* H GGATGTCCATATTAGGACATCTGCGTCA

CRE TGACGTCA

B

Origin	Sequences	References
c-*fos*	GGATGTCCATATTAGGACATCT	a
X. actin	AGATGCCCATATTTGGCGATCT	b
Krox-20.1	CTCAGTCCATATATGGGCAGCG	c
Krox-20.2	TCTTCTCCTTTTTTGGAAAGTC	c
Krox-24.1	TGCTTCCCATATATGGCCATGT	d
consensus	XG(A/C)TG(T/C)CCATAT(T/A)TGG(A/C)CA(T/G)CT	

FIG. 6. Comparison of SRE sequences. (A) Comparison of the SRE region of the human c-*fos* gene (positions −320 to −293) with the homologous region of *Krox-20* (positions 228 to 279). The SRE- and CRE-like palindromes of *Krox-20* are indicated (arrows), as is the neighboring half SRE-like sequence. Asterisks indicate identical nucleotides between c-*fos* (or the CRE core motif) and *Krox-20*. (B) Comparison of known SRE sequences. SRE sequences derived from the human c-*fos*, *Xenopus laevis* cytoskeletal actin, mouse *Krox-20*, and *Krox-24* genes were compared. A consensus sequence was derived. In the consensus sequence, the underlined nucleotides are conserved in all five sequences. The other nucleotides correspond to conservation in at least three sequences. References are as follows: a, references 67 and 58; b, reference 50; c, this work; d, Janssen-Timmen et al., in preparation.

Two chemically synthesized oligonucleotides corresponding to the putative *Krox-20* SRE, either alone or in combination with the putative *Krox-20* CRE-like sequence, and a 124-bp *Alu*I DNA restriction fragment (Fig. 3, positions 206 to 330) containing the *Krox-20* SRE- and CRE-like sequences as well as the downstream SRE-like partial sequence, were cloned in both orientations upstream from a human c-*fos* gene lacking the 5′-flanking sequences upstream of position −261 and therefore deleted of its own SRE (68). This deleted c-*fos* gene was shown to be unresponsive to serum when transfected into NIH 3T3 cells, but its serum inducibility could be restored by reintroducing the c-*fos* SRE (67, 68). The different c-*fos* derivatives were subjected to a transient serum stimulation assay (67, 68). Our results indicated that all three sequences were able to substitute functionally for the c-*fos* SRE when inserted in their natural orientations (Fig. 7). The induction ratios were on the order of 50-fold, as measured by densitometer scanning of the resulting autoradiograms. The three sequences also functioned when cloned in the reversed orientation, and the kinetics of induction of the human c-*fos* gene in all the constructs tested was identical to that of a wild-type c-*fos* gene construction containing 711 bp of 5′-flanking sequences (data not shown). Therefore, we conclude that the shortest *Krox-20*-derived sequence contains an SRE with an activity similar to that of the c-*fos* SRE.

Subsequently, a sequence with weaker similarity to the c-*fos* SRE was noted upstream of the *Krox-20* transcription initiation region, between positions −74 and −53 (Fig. 3 and 6B). An oligonucleotide carrying this sequence was also tested in the transient serum stimulation assay after insertion in front of the deleted c-*fos* gene. The presence of this oligonucleotide led to weak (5- to 10-fold) but reproducible induction in both orientations (Fig. 7 and data not shown). Thus, the *Krox-20* gene contains two SRE sequences which are most likely the *cis*-acting regulatory elements responsible for the serum-dependent transcriptional activation of the gene.

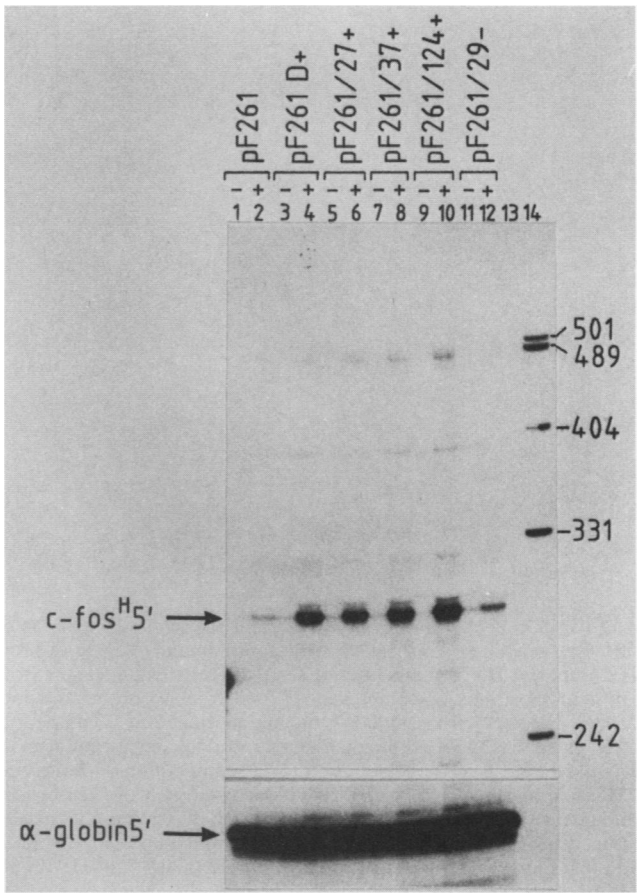

FIG. 7. A synthetic *Krox-20* SRE-like sequence restores inducibility to an uninducible c-*fos* gene. Total RNA was extracted from NIH 3T3 cells cotransfected with the human alpha-1-globin gene and derivatives of the human c-*fos* gene. RNase mapping of the 5′ end of both mRNAs was carried out on 40 μg of RNA with uniformly labeled SP6 RNA probes. The digestion products were analyzed by electrophoresis on a 5% polyacrylamide sequencing gel. Arrows indicate the positions of the protected fragments corresponding to the 5′ end of the mRNAs. The name of the c-*fos* derivative genes used in the transfection are indicated above the lanes (see Materials and Methods for the description of the constructs). Lanes 1 to 12, − and + indicate that the RNA was collected from quiescent cells or cells stimulated by serum for 45 min, respectively; lane 13, analysis of 40 μg of tRNA; lane 14, DNA size marker.

***Krox-20* and c-*fos* SREs bind the same cellular factor.** The identification of SREs within or close to the *Krox-20* gene raised the possibility that these *cis*-acting sequences might bind the cellular factor, the SRF, which interacts with the related c-*fos* SRE. We tested this possibility by using a DNA binding gel electrophoresis assay (26, 28, 65, 68). The 124-bp *Alu*I DNA fragment containing the SRE and the CRE-like motif of *Krox-20* was labeled with ^{32}P and incubated with a HeLa cell nuclear extract. Protein-DNA complexes were separated from unbound probe by electrophoresis on polyacrylamide gels as described previously (68). A major complex band was resolved (Fig. 8, lane 2). We evaluated the relative affinities of different SREs and unrelated DNA sequences for the factor present in the complex by competition assay with the *Alu*I fragment. Oligonucleotides containing either *Krox-20* SRE-1 or c-*fos* SRE competed equally efficiently with the *Alu*I DNA fragment, while an oligonucleotide containing a binding site for the transcription factor

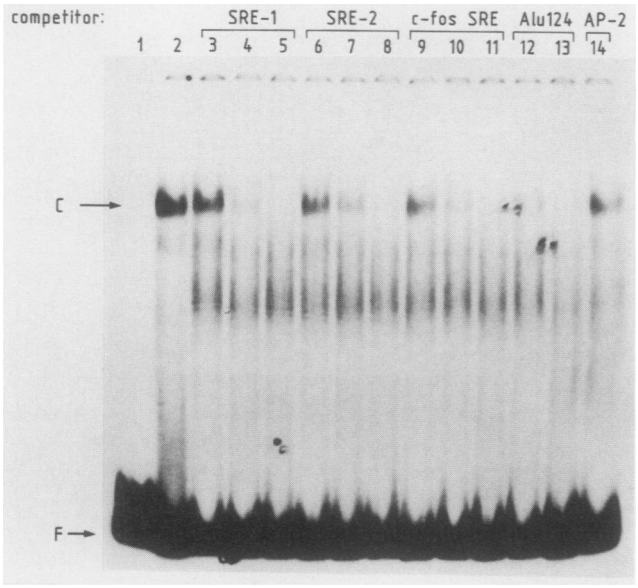

FIG. 8. *Krox-20* and c-*fos* SREs bind the same cellular factor. Binding reactions were set up with approximately 0.2 ng of probe (124-bp *Alu*I DNA fragment) and 12 μg of HeLa cell nuclear extract protein. Positions of complexed (C) and free (F) probes are indicated. Lanes: 1, probe alone; 2, no competition; 3 to 5, competition with the *Krox-20* SRE-1 oligonucleotide; 6 to 8, competition with the *Krox-20* SRE-2 oligonucleotide; 9 to 11, competition with the c-*fos* SRE oligonucleotide; 12 and 13, competition with the 124-bp *Alu*I fragment; 14, competition with the AP-2 binding site oligonucleotide. Competitor DNA represented a 10-fold (lanes 3, 6, and 9), 30-fold (lanes 4, 7, 10, and 12), and 100-fold (lanes 5, 8, 11, 13, and 14) molar excess over probe DNA.

AP-2 did not compete (Fig. 8, lanes 3 to 5, 9 to 11, and 14). Surprisingly, the oligonucleotide containing the weak *Krox-20* SRE-2 competed almost as efficiently as SRE-1 (Fig. 8, lanes 6 to 8). The band observed above the probe in the absence of nuclear extract (Fig. 8, lane 1) was presumably due to partial denaturation of the probe. This denatured DNA was not involved in specific complex formation, as shown by the absence of competition observed with single-stranded oligonucleotides (data not shown). A reversed experiment was performed in which the c-*fos* SRE oligonucleotide was labeled and subjected to the DNA binding gel electrophoresis assay. Consistent with the results of Treisman (68), a single complex band was detected (data not shown). This labeled complex could be outcompeted with equal efficiency by the c-*fos* SRE and the *Krox-20* SRE-1 (data not shown). We conclude from these experiments that, at least in vitro, the c-*fos* SRE and the *Krox-20* SRE-1 bind the same cellular factor with similar affinities and that this factor is likely to be identical to the SRF described by Treisman (68).

DISCUSSION

In this paper, we describe the genomic structure of *Krox-20*, an immediate-early serum response gene, which encodes a putative transcription factor. We show that *Krox-20* RNA maturation results in two mRNAs which differ by the presence of a short intron and that the putative transcription factors encoded by the two mRNAs would differ at their N termini. In serum-stimulated fibroblasts, the spliced mRNA is present in very small amounts and it may not play any role. However, the gene is also expressed in a tissue-specific manner during development, mainly in the thymus in the adult (10) and in specific regions of the nervous system in the embryo (D. Wilkinson et al., submitted). Therefore, it is possible that in these situations the spliced mRNA represents a larger proportion of total *Krox-20* RNA and that the shorter protein plays a functional role.

Krox-20 has been localized on chromosome 10 both in humans and in the mouse. This is in contrast to the closely related gene *Krox-24/Egr-1*, which was localized on human chromosome 5 (66; Janssen-Timmen et al., manuscript submitted) and on mouse chromosome 18 (Janssen-Timmen et al., submitted). Therefore, although the two genes encode almost identical zinc fingers (42), they are not closely located in either genome.

***Krox-20* and c-*fos* are coregulated.** We have shown previously that c-*fos*, *Krox-20*, and *Krox-24* have very similar kinetics of activation in NIH 3T3 fibroblasts following stimulation by serum or purified growth factors (10, 42). Our data now suggest that the basis for this similarity is the existence of common activation mechanisms. *Krox-20* and c-*fos* have similar *cis*-acting regulatory sequences which appear to be functionally interchangeable and to bind the same cellular protein, which is likely to be identical to the SRF described by Treisman (68). The combination of the SRE and of its cognate protein therefore presumably constitutes the molecular basis for at least part of the coregulation of the two genes in fibroblast cells. In addition, this coregulation is likely to be of a very general significance and to apply to a number of genes that are part of the immediate-early response to serum stimulation. Indeed, we have shown that *Krox-24* activation also relies on the same *cis*- and *trans*-acting elements (Janssen-Timmen et al., submitted). The *Xenopus laevis* cytoskeletal actin gene, which is activated during G0/G1 transition after transfection into mouse cells, although with different kinetics, also contains a functional SRE sequence (50). In conclusion, the SRE-SRF couple appears to have been highly conserved during evolution and to constitute a central branch point in the regulatory pathway of cellular response to growth factors.

Particularities of *Krox-20* SREs. Figure 6B presents a comparison of SRE sequences derived from four serum-inducible genes. Comparison of their sequences suggests that a motif essential for activity is 5′-CCA/TTATA/TA/TGG-3′. This is consistent with dimethyl sulfate protection and interference experiments, which indicated that the G residues of this motif were essential contact points between the SRF and the SRE (50). In the case of *Krox-20*, we have identified two SREs. Although the 5′ one, SRE-2, appears to be of weaker activity, it might nevertheless contribute to the regulation of the gene. This is consistent with our observation that a hybrid gene consisting of the 5′-flanking region of *Krox-20* up to the *Eco*RI site (positions −1378 to −41 [Fig. 3]), fused to the coding sequence of the bacterial chloramphenicol acetyltransferase gene, is slightly inducible by serum (two- to threefold) after transient transfection into NIH 3T3 cells (data not shown). This construction retains the SRE-2 but not the SRE-1. The sequence of the SRE-2 is relatively different from those of the other SREs whose activities have been tested so far (Fig. 6B). The absence of alternating A and T residues and in particular of the conserved central AT dinucleotide might be responsible for its weaker activity. Another particular feature of *Krox-20* is the fact that the SRE-1 is located within an exon (Fig. 2 and 3). In addition, the SRE-1 sequence is followed by a 9-bp sequence complementary to its 3′ half (Fig. 6A). Therefore,

it is possible that the complementary nucleotides anneal within the *Krox-20* RNA and that this secondary structure plays a regulatory role at a posttranscriptional level.

Possible roles for the *Krox-20* CRE-like sequence. A striking observation is that the region of similarity between c-*fos* and *Krox-20* extends 3′ to the SREs and includes a sequence homologous to the CRE core motif (Fig. 6A). Even more striking is the fact that there is also a CRE-like sequence 3′ to the most 3′ SRE of *Krox-24* (Janssen-Timmen et al., submitted). The spacing between SRE and CRE sequences is identical in the three genes (Fig. 6A and data not shown). This strong conservation suggests a regulatory role for this sequence. In the case of c-*fos*, the gene is known to be regulated by cyclic AMP in a tissue-specific manner (6). However, involvement of the CRE-like sequence in this regulation has not been reported. We do not know whether *Krox-20* or *Krox-24* gene expression is regulated by cyclic AMP.

Another possible role for the CRE-like sequence is indicated by an array of indirect observations. (i) Analysis of the transcription of c-*fos* and *Krox-20* following serum stimulation in the presence or absence of cycloheximide indicates that transcriptional activation is transient and that cycloheximide prolongs the period of transcription (1). This finding is consistent with the idea that a negative regulator of c-*fos* and *Krox-20* transcription is coactivated with them following serum stimulation. (ii) The CRE-like sequences of c-*fos* and *Krox-20* resemble the consensus sequence for the binding site of the human transcription factor AP-1: 5′-C/GTGACTC/AA-3′ (3, 40, 41). Indeed, the c-*fos* CRE-like sequence has been shown to bind the mouse homolog of AP-1 (55). (iii) The c-*jun* gene, which encodes one of the proteins constituting AP-1 (2, 5), is activated in parallel to c-*fos* and *Krox-20* during serum stimulation (58, 62). (iv) The products of c-*fos* and c-*jun* have been shown to be part of a complex which might be involved in the repression of the expression of c-*fos* itself and of the adipocyte gene *aP2*, via AP-1 binding sites (20, 58, 63). Therefore, it is possible that the CRE-like motif is involved in transcriptional repression of *Krox-20* by interacting with the products of c-*jun* and/or c-*fos* or with the products of closely related genes.

ACKNOWLEDGMENTS

We thank E. Passage for excellent technical assistance, A.-M. Frischauf for the gift of the cosmid libraries, R. Treisman for the gift of plasmids and helpful discussions, T. Pohl for the gift of terminase and for help in using the REMA program for restriction mapping, A. Lamond for the gift of HeLa cell nuclear extract, D. Wilkinson and M. Bucan for the communication of unpublished information, and R. Cortese, S. Ness, and M. Yaniv for critical reading of the manuscript.

P.C., U.J.-T., and M.Z. were supported by fellowships from the Association pour la Recherche sur le Cancer, the Deutsche Forschungsgemeinschaft, and the European Molecular Biology Laboratory, respectively.

ADDENDUM IN PROOF

The nucleotide sequence of a cDNA likely to correspond to the human homolog of *Krox-20* has recently been reported (Joseph et al., Proc. Natl. Acad. Sci. USA **85**:7164–7168, 1988).

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